Distinct Susceptibilities of Corneal Pseudomonas aeruginosa Clinical Isolates to Neutrophil Extracellular Trap-Mediated Immunity

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Ocular bacterial keratitis, often associated with Pseudomonas aeruginosa bacterial infection, commonly occurs in contact lens wearers and may lead to vision impairment. In this study, we analyzed the contribution of neutrophil extracellular traps (NETs) to the mediation of protection during ocular keratitis. Both invasive and cytotoxic P. aeruginosa clinical isolates induced NET release by neutrophils. NETs carried the characteristic histone proteins, elastase, lysozyme, myeloperoxidase, and metabolic enzymes. While the invasive P. aeruginosa strains PAO1 (serogroup O5) and 6294 (serogroup O6) were trapped by NETs, the cytotoxic P. aeruginosa strains 6077, 6206 (serogroup O11), and PA14 (serogroup O10) were less sensitive to NET capture. The mechanism of escape by the cytotoxic strains from adhesion to NETs involved the shedding of outer membrane vesicles (OMVs) that outcompeted the cytotoxic P. aeruginosa strains for NET binding. When ocular infection was caused by an invasive strain in vivo, NETs were released at the ocular surface to capture bacteria, limiting their spread. Treatment with MNase I had a dose-dependent effect, with low doses of MNase speeding up bacterial clearance and high doses of MNase having toxic consequences. Cumulatively, our data suggest that NET-mediated immunity is a two-step process. Initially, pathogens attach to NET fragments; subsequently, upon nuclease activity, active serine proteases, which proteolytically degrade NET-associated proteins and promote DNase activity, are released. Therefore, a balance between NET production and NET degradation is needed to achieve maximal NET immunity.

Infections caused by Pseudomonas aeruginosa are frequently associated with bacterial ocular keratitis, a condition that carries the risk of vision impairment and occurs in contact lens wearers or after ocular trauma. The majority of bacterial clinical isolates derived from corneal ulcers arising during keratitis can be divided into two functionally distinct groups: invasive and cytotoxic isolates. These two groups have distinct phenotypes; the cytotoxic strains cause rapid cell lysis, whereas the invasive strains replicate in the target cells (1, 2). These differences translate into various severities of disease; in mouse keratitis studies where infection is induced by cytotoxic strains, disease pathology is worse than that caused by invasive strains (3). Consistent with these findings, patients infected with cytotoxic strains have experienced more severe pathology and, consequently, suffered from greater visual impairment after recovery from infection (4). The immune response to both invasive and cytotoxic strains is dominated by dense neutrophil infiltrates, yet the infiltration pattern and responses of polymorphonuclear neutrophils (PMNs) differ depending on the type of strain. Due to the recently described ability of neutrophils to release DNA in the form of neutrophil extracellular traps (NETs) (5) and the presence of extracellular DNA during cytotoxic infections, we questioned whether the cytotoxic strains trigger NETosis (5).

To address this question, we used proteomic approaches to generate a comprehensive list for P. aeruginosa-triggered NET proteomes released in response to either invasive or cytotoxic challenges. We report that the cytotoxic strain PA14 induced release of NET eDNA that was in complex with the characteristic NET proteins. Because NETs contain a range of antimicrobial factors (e.g., histones, defensin, lysozyme, and myeloperoxidase), it is expected that they have bactericidal and fungicidal properties (5). While Escherichia coli, Klebsiella pneumoniae, and Candida albicans were reported to be sensitive to NET-mediated killing, Streptococcus pneumoniae, Haemophilus influenzae, Streptococcus pyogenes, Neisseria meningitides, Mycobacterium tuberculosis, Staphylococcus aureus, Staphylococcus epidermidis, and Yersinia pestis were shown to be resistant (6–12). Whether NETs trap and kill P. aeruginosa is controversial. We, along with others, reported that the nontuberculous cystic fibrosis-derived P. aeruginosa strains were sensitive to NET trapping while the mucoid strains were resistant (13–15). In contrast, Parker et al. reported that P. aeruginosa was not sensitive to NET-mediated killing (16). Given that the keratitis-associated P. aeruginosa clinical isolates show distinct population characteristics and express virulence factors different from those of the pneumonia isolates, a separate study analyzing their sensitivities to NET capture and killing is warranted (17). Here, we compared the susceptibility of invasive versus cytotoxic P. aeruginosa clinical isolates to NET-mediated capture and killing. We found that invasive strains were trapped by NETs and were weakly susceptible to NET-mediated killing, whereas the cytotoxic strains were less sensitive to NET capture.

MATERIALS AND METHODS

Bacterial strains. The invasive P. aeruginosa strains PAO1, 6294, and 6354 and the cytotoxic P. aeruginosa strains PA14, 6077, 6073, and 6206 were used throughout these experiments. The PAO1 AKA1401 and PAO1

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pLSP2 strains were provided by G. Pier (BWH) (18). The PAO1 ExoU strain was provided by S. Lory (HMS).

**Mice.** All animal studies were performed in accordance with guidelines established by the Harvard Medical School Animal Care and Use Committee. The experimental protocols were approved by the Institutional Animal Care and Use Committee of the Harvard Medical Area Office for Research Subject Protection and were consistent with the Association for Research in Vision and Ophthalmology guidelines for studies in animals. Infections were carried out as described previously (19). Mice were anesthetized with ketamine and xylazine injections. Three 0.5-cm scratches were made on the corneas, and an inoculum of *P. aeruginosa* 6294 (1 × 10⁶ CFU/eye) was delivered in 10 µl onto the eye. Mice remained sedated for about 30 min. For evaluation of corneal pathology, daily scores were recorded by an observer who was unaware of the experimental status of the animals. Pathology scores were based on a scoring system using a graded scale of 0 to 4. A score of 0 indicated that the eye was macroscopically identical to the uninjected contralateral control eye. A score of 1 indicated faint opacity partially covering the pupil. A score of 2 indicated dense opacity covering the entire anterior segment, and a score of 4 indicated the perforation of the cornea, phthisis bulbi (shrinkage of the globe after inflammatory disease), or both. To determine the levels of bacteria in the cornea 24 or 48 h after infection, mice were sacrificed, the eyes were enucleated, and corneas were dissected from the ocular surface. To quantify extracellular levels of *P. aeruginosa*, corneas were excised, suspended in phosphate-buffered saline (PBS), and vortexed. Subsequently, serial dilutions were made and plated on *P. aeruginosa*-selective cetrime plates.

Increasing concentrations of MNI I (Sigma), ranging from 300 to 5,000 U/cornea, were applied topically every 8 h after the infectious challenge. PBS (MNI I solvent) was administered to the control group.

**Isolation of primary human neutrophils.** PMNs were isolated from healthy human donors. Blood (10 ml) was drawn from healthy individuals, with their informed consent, using a sodium-heparin blood collection kit (Becton Dickinson Vacutainer Safety-Lok blood collection set). Blood was inverted to mix with the anticoagulant agent. A density gradient was prepared using Polymorph reagent (Axis-Shield) by following instructions provided by the manufacturer. The blood was layered on the gradient and centrifuged at 300 × g for 30 min, at room temperature, using a swing-bucket centrifuge without braking. Purified PMNs were resuspended in 5 ml of Hanks balanced salt solution buffer without Ca²⁺ or Mg²⁺ ions (HBSS⁻/⁺, Invitrogen, CA). Immediately prior to adding bacteria, PMNs were pelleted at 400 × g for 10 min and resuspended in HBBS with Ca²⁺, Mg²⁺, and 0.1% gelatin (HBSS⁻/⁺, Invitrogen).

**Isolation of primary murine neutrophils.** Bone marrow was flushed from femurs and tibias of 6- to 8-week-old macrophage inhibitory factor was provided by G. Pier (BWH) (18). The PAO1 ExoU strain was provided by the Harvard Medical School Animal Care and Use Committee. The experimental protocols were approved by the Institutional Animal Care and Use Committee of the Harvard Medical Area Office for Research Subject Protection and were consistent with the Association for Research in Vision and Ophthalmology guidelines for studies in animals. Infections were carried out as described previously (19). Mice were anesthetized with ketamine and xylazine injections. Three 0.5-cm scratches were made on the corneas, and an inoculum of *P. aeruginosa* 6294 (1 × 10⁶ CFU/eye) was delivered in 10 µl onto the eye. Mice remained sedated for about 30 min. For evaluation of corneal pathology, daily scores were recorded by an observer who was unaware of the experimental status of the animals. Pathology scores were based on a scoring system using a graded scale of 0 to 4. A score of 0 indicated that the eye was macroscopically identical to the uninjected contralateral control eye. A score of 1 indicated faint opacity partially covering the pupil. A score of 2 indicated dense opacity covering the entire anterior segment, and a score of 4 indicated the perforation of the cornea, phthisis bulbi (shrinkage of the globe after inflammatory disease), or both. To determine the levels of bacteria in the cornea 24 or 48 h after infection, mice were sacrificed, the eyes were enucleated, and corneas were dissected from the ocular surface. To quantify extracellular levels of *P. aeruginosa*, corneas were excised, suspended in phosphate-buffered saline (PBS), and vortexed. Subsequently, serial dilutions were made and plated on *P. aeruginosa*-selective cetrime plates.

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a 13.2-ml thin-wall polyallomer ultracentrifuge tube (Beckman Coulter), overlaid with 2 ml of 30% sucrose (30%, wt/vol, sucrose in HBSS−/− supplemented with 2X protease inhibitor cocktail). The third sucrose layer of 2 ml of 10% sucrose was added, followed by 2 ml of infection reaction supernatants. Assembled gradient samples were ultracentrifuged at 1,600 × g for 22 h at 4°C using an SW41 rotor in a Beckman L8-M ultracentrifuge. Samples were fractionated with a peristaltic pump into 10 1-ml fractions per gradient tube. Fractions were analyzed for DNA via PicoGreen assays and for protein content via Bradford assays (Bio-Rad). Nonstimulated cells were used as background controls.

The protein in each sample was precipitated with trichloroacetic acid (TCA) overnight at −20°C. Supernatants were carefully removed and washed twice with 100% acetone. The frozen pellets were processed for mass spectrometry analysis at the Taplin Biological Mass Spectrometry Core Facility (Harvard Medical School, Boston, MA). The functional analysis of the proteins identified by liquid chromatography-tandem mass spectrometry (LC-MS/MS) was carried out using PANTHER classification and visualized using String v9.05.

Confocal imaging. Corneal washes were cytospun onto lysine-coated slides, fixed with 4% formalin, blocked with 5% goat serum-PBS for 1 h, and stained with anti-mouse histone H4 rabbit IgG (Santa Cruz) overnight at 4°C; slides were washed and developed with goat anti-rabbit Alexa 586 (Invitrogen) and counterstained to visualize DNA with Sytox Green (Invitrogen), per the manufacturer’s instructions. Samples were imaged using an LSM 510 META laser scanning confocal microscope (Zeiss, Germany). Alexa 488 was detected using the 488-nm excitation line and a 500- to 530-nm-bandpass filter. Alexa 555 and Alexa 647 were excited at the wavelengths of 543 nm and 633 nm and detected using a 560- to 615-nm-bandpass filter and a 650-nm-long-pass filter, respectively.

OMV production. *P. aeruginosa* was grown in LB broth until the OD550 reached 1.2 to 1.6 nm. Cells were removed by centrifugation at 6,600 × g for 10 min. Supernatants were collected and filtered through a 0.22-μm Durapore polyvinylidene difluoride (PVDF) filter (Millipore) to remove any remaining bacteria. Outer membrane vesicles (OMVs) were obtained from the cell-free supernatants by centrifugation at 39,000 × g for 1 h and resuspended in HBSS−/− as described previously (21, 22). OMVs were quantified based on protein levels per density of bacterial culture and visualized using transmission electron microscopy core facility services at the Harvard Medical School core facility.

Statistical analysis. Data were analyzed with GraphPad Prism 5.0 software (GraphPad Software Inc.). Sample distributions were analyzed for normality. When appropriate, data were compared using Student’s t test or 1-way analysis of variance (ANOVA), followed by Bonferroni’s posttest. P scores of less than 0.05 were considered significant. Proteomic data were compared using chi-squared test. Significance was reported when P values were lower than 0.05.

RESULTS

Cytotoxic *P. aeruginosa* strains stimulate NET release more than invasive strains. We tested the ability of keratitis-associated *P. aeruginosa* clinical isolates (the invasive PA01 and 6294 strains and the cytotoxic PA14 and 6077 strains) to stimulate NET formation (Fig. 1; also see Fig. S1 in the supplemental material). At low MOI, no significant differences were observed in the ability of the strains to trigger eDNA release. At an MOI of 10, the cytotoxic strains 6077 (Fig. 1A) and PA14 (Fig. 1B) induced significantly increased levels of NETosis compared to the invasive 6294 and PA01 strains. To determine whether those differences were attributed to effector proteins translocated via the type III secretion system, the PA01 strain that expressed ExoU was used to stimulate NETosis and compared to the parental PA01 strain or the PA14 strain (Fig. 1A). ExoU presence conferred an increase in eDNA release.

To verify that the measured eDNA carried the specific markers of NET fibers, NET fragments were released with partial endonuclease (e.g., MNase) cleavage to generate high-molecular-weight (HMW) DNA fragments, and the samples were fractionated over the sucrose density gradient. The individual fractions were analyzed for DNA size, concentration, and neutrophil elastase (NE), a NET protein marker (Fig. 1C and D) (23). Fractions 1 to 5 contained HMW DNA (>1 kb), whereas fractions 6 to 9 contained low-MW DNA (<1 kb). The NE signal was enriched in the HMW DNA-rich fractions of the PA14 and PA01 ExoU-stimulated samples, indicating the presence of NETs. In contrast, the levels of eDNA released in response to the PA01 stimulation were minimal, and consequently, the NE signal was low, illustrating that the cytotoxic strain PA14 or ExoU-carrying strain was more efficient at triggering NETosis. These studies were followed by proteomic analysis of NET-associated proteins (see Fig. S2 in the supplemental material). Twenty-eight proteins were shared between the PA01 strain and the PA14 strain (see Table S1 in the supplemental material). Many of these proteins were identical to previously reported NET-associated proteins, confirming the ability of PA14 to induce NETosis (13, 24, 25).
To functionally prove that the cytotoxic PA14 strain induced NETosis, separate experiments were carried out with differentiated HL-60 cells (used as a model system for neutrophil responses) exposed to P. aeruginosa PA14 at an MOI of 10, and cellular supernatants were acetone precipitated and resolved on 4 to 15% NuPAGE, followed by LC-MS/MS identification of the differentially present proteins. The plus sign indicates samples that received DNase I treatment during infection, whereas the minus sign denotes nondigested samples. The proteins were identified by LC-MS/MS and are listed next to the bands that were excised for sequencing.

To determine whether NETs were produced spontaneously, or during NETosis by P. aeruginosa-induced keratitis, the ocular surface eDNA was quantified and characterized to determine whether complexes were formed with bacteria. A significant increase in the eDNA was seen at the ocular surface at 48 h after the onset of infection (Fig. 6). The trapping of PAO1 was significantly inhibited by addition of PA14-derived OMVs (Fig. 5C). Cumulatively, these data suggest that the cytotoxic PA14 strains escape capture by NETs by releasing OMVs. Notably, the cytotoxic strains secreted higher OMV levels than did the invasive strains even at steady state (Fig. 5D).

**NETs are released at the ocular surface to trap bacteria.** Despite the observation that P. aeruginosa induced NETosis in vitro, it was not clear whether NETosis occurred during infection in vivo. To determine whether NETs were produced in vivo during P. aeruginosa-induced keratitis, the ocular surface eDNA was quantified and characterized to determine whether complexes were formed with bacteria. A significant increase in the eDNA was seen at the ocular surface at 48 h after the onset of infection (Fig. 6). Cytospins of ocular washes showed abundances of clustered bac-

**FIG 2** NET DNA stabilizes NET protein composition. Differentiated HL-60 cells were exposed to P. aeruginosa PA14 at an MOI of 10, and cellular supernatants were acetone precipitated and resolved on 4 to 15% NuPAGE, followed by LC-MS/MS identification of the differentially present proteins. The plus sign indicates samples that received DNase I treatment during infection, whereas the minus sign denotes nondigested samples. The proteins were identified by LC-MS/MS and are listed next to the bands that were excised for sequencing.

**OMVs inhibit P. aeruginosa adhesion to NETs.** To gain insights into the potential mechanism of resistance of the cytotoxic strains to NET capture, the NET-associated bacterial PA14-derived proteome was analyzed. One hundred forty-six bacterially derived proteins copurified with NETs. These proteins could be separated into distinct groups depending on function, including monosaccharide metabolism, protein folding, and protein metabolism (Fig. 5; also see Table S3 in the supplemental material). Based on these findings, we hypothesized that the P. aeruginosa PA14-derived exoproteome competes with bacterial binding to NETs. Prior studies revealed that P. aeruginosa spontaneously, or under stress, produces OMVs (29). When comparing the recently identified P. aeruginosa PA14 OMV proteome (30) to the NET-associated proteome, 81 proteins were shared (Fig. 5B). This indicated a significant degree of commonality (P < 0.0001 by chi-square test). To determine whether P. aeruginosa-produced OMVs competed for NET binding, PA14-derived OMVs were mixed at increasing concentrations with the sensitive strain PAO1. The trapping of PAO1 was significantly inhibited by addition of PA14-derived OMVs (Fig. 5C). Cumulatively, these data suggest that the cytotoxic PA14 strains escape capture by NETs by releasing OMVs. Notably, the cytotoxic strains secreted higher OMV levels than did the invasive strains even at steady state (Fig. 5D).

**Distinct susceptibilities of P. aeruginosa strains to NET capture and killing.** Based on the proteomic profile of NETs, the expectation was that NETs carry antimicrobial activity. The bactericidal properties of NETs have attracted scientific interest; however, the question of whether NETs are capable of trapping or killing P. aeruginosa remains contentious (14, 16, 28). To determine the functional significance of NETs, in vitro experiments addressing NET-mediated capture of P. aeruginosa and killing were carried out. In these experiments, neutrophils were pretreated with PMA to induce equal levels of NETosis and exposed to either invasive (e.g., PAO1 or 6294) or cytotoxic (e.g., 6206 or PA14) P. aeruginosa strains in the presence or absence of endonuclease (e.g., MNase) (Fig. 3). These experiments suggested that NETs aggregated and killed 50% of the invasive strains PAO1 (serogroup O5/O2) and 6294 (serogroup O6), whereas NETs failed to kill the cytotoxic strains 6206 and 6077 (serogroup O11) and PA14 (serogroup O10) (Fig. 3).

To determine whether the cytotoxic strains escaped NET capture because of changes in the LPS outer core polysaccharide structures, the previously described mutant PAO1 AKA1401 strain that expressed partial B-band LPS was analyzed for NET capture and killing. This strain was captured 25% better than the parental PAO1 strain and was sensitive to NET killing (Fig. 3 and 4). These data illustrated that strains with an incomplete O-antigen were slightly more sensitive to NET capture. In a separate series of experiments, a P. aeruginosa PAO1 strain, engineered to express the serogroup O11, termed PAO1R pLPS2 (18), was analyzed for sensitivity to NET-mediated capture and killing. This strain was captured and killed by NETs at 100 or 200 min after exposure to NETosising neutrophils, demonstrating that altering the B-band structure was not sufficient to confer resistance to NETs and did not explain the resistance to NET capture by the cytotoxic strain 6077 or 6206, which belonged to the O11 serogroup (Fig. 4B).
bacteria attached to the surfaces of NET eDNA fragments covered with histones (Fig. 6).

To define the biological significance of NETs, exogenous MNase I was applied topically every 8 h after the induction of infection. Topical application of low doses of MNase I decreased bacterial burden and disease pathology, illustrating that the dose of administered MNase I should be carefully monitored, since topical application of high levels of MNase I had toxic effects (Fig. 7). Consequently, high concentrations of MNase I resulted in the worsening of disease symptoms.

FIG 3 Invasive (6294 and PAO1) and cytotoxic (6206 and PA14) P. aeruginosa strains show distinct sensitivities to NET-mediated capture and killing. Purified human PMNs were pretreated with PMA to induce NETosis and exposed to 6294, PAO1, 6202, and PA14 at an MOI of 0.1 for 100 min. The presence of endonuclease in the reaction mixture is indicated with a plus sign. Results are representative of two independent experiments. Samples were compared using one-way ANOVA for variance, and significant differences are indicated with asterisks.
DISCUSSION

P. aeruginosa-induced infections have different immune characteristics due to the distinct virulent systems utilized by various strains. P. aeruginosa harbors several secretion systems that translocate effector molecules into host target cells or secrete effectors into the extracellular milieu. In addition, there are about 20 different serogroups denoting differences in the expression of LPS-O antigens, which are key antigenic determinants (31). Therefore, P. aeruginosa is challenging to handle therapeutically. To define novel therapeutic approaches, comprehensive comparative stud-
ies that characterize the strain-specific innate and adaptive immunity are needed. In this study, we evaluated the ability of the invasive versus cytotoxic strains to induce NETosis and their relative susceptibilities to NET capture. We found that all strains induced NETosis in vitro. Notably, the cytotoxic strains (e.g., PA14 and 6077) were better at stimulating NETosis than the invasive strains (e.g., PAO1 and 6294), a phenomenon due, at least in part, to ExoU secretion. The cytotoxic strain-induced NETosis was characterized by the typical protein assembly of DNA fibers that included MPO, /H251-enolase, thymidine kinase (TK), etc. (see Table S1 in the supplemental material), as well as active NE (Fig. 2). The latter was released by limited nuclease treatment and proteolytically degraded NET-associated proteins, thereby facilitating NET clearance. Although the cytotoxic strains were more efficient at inducing NETosis, they were significantly less sensitive to capture, illustrating that these strains hijacked PMN responses by triggering an ineffective neutrophil response.

The distinction, highlighted by this study, between invasive and cytotoxic strains of Pseudomonas aeruginosa raises the significant issue of NET evasion mechanisms that are utilized by pathogens. This study demonstrated that cytotoxic Pseudomonas aeruginosa strains were able to evade containment by NETs. These strains, however, are not unique in this capability. For instance, a similar strategy was utilized by N. meningitides to evade capture by NETs (7). These observations suggest that novel therapeutics should target pathways responsible for OMV secretion. Clearly, the identities of the molecular pathways that are induced by PMNs and result in shedding of OMVs by P. aeruginosa should be further characterized to enable pathway-specific targeting.

These studies have implications for vaccine design and therapy. The NET-associated bacterial proteome data have implications for vaccine development. For example, we identified the significant presence of OprF and OprI attached to NETs, illustrating that adhesion to bacteria or P. aeruginosa-derived OMVs is facilitated through these proteins. The outer membrane proteins OprF and OprI are abundant outer membrane porins present on the surface of bacteria and are implicated in OMV formation (30). Due to their immunogenicity and key bacterial functions, OprF and OprI are vaccine candidates against P. aeruginosa-initiated infections (32, 33). Current vaccine efforts that utilize recombinant protein composed of OprF and OprI epitopes show that the recombinant vaccine is immunogenic and well tolerated in humans (32). Our data strengthen the argument that OprF and OprI contribute to the pathogenicity of P. aeruginosa by revealing a novel aspect of OprF and OprI activities, particularly bacterial adhesion.

In addition to OprF and OprI, the remainder of the identified bacterial NET proteome may yield novel vaccine targets. Some of the most abundant proteins adherent to NETs were GroEL, GroES,
phosphoglycerate kinase (PGK), DnaK, elongation factor Tu, and elongation factor Ts. Typically, these proteins have intracellular metabolic or chaperone functions, but there is evidence that, when present at the surface, they promote bacterial adhesion (34). For example, DnaK (Hsp70) is a cell surface protein in Listeria monocytogenes, Neisseria meningitides, and Mycobacterium tuberculosis that binds plasminogen, whereas GroEL promotes adhesion of Enterococcus faecalis to actin or actin binding proteins (35–37). Interestingly, monoclonal antibody to H. pylori GroEL inhibits the growth of the bacterium, suggesting that there is a connection between the growth control and surface localization of this protein (38). It is likely that antigen-specific immunity against these adhesion-mediating proteins interferes with bacterial or bacterially derived OMV deposition onto host surfaces.

Our research has direct implications for therapy. Human DNase I, or dornase alfa, has been approved since 1994 for use in cystic fibrosis patients to solubilize dense DNA-containing cystic fibrosis sputum (26, 39). Our work expands the applicability of recombinant human DNase by demonstrating that nuclease treatment can be used as an adjunctive treatment strategy during acute keratitis to expedite bacterial clearance. However, the levels of administered nuclease should be controlled, as treatment with high levels of DNase has a negative impact on bacterial clearance, most likely through the release of NET-associated active serine proteases like NE.

FIG 6 Ocular surface eDNA increases during ocular keratitis. (A) eDNA was quantified 48 h after exposure to P. aeruginosa 6294. (B) The ocular surface was washed with sterile PBS, and cells were cytopspun and stained for the presence of DNA and histone H4. Bacterial clusters on the NETs are indicated with grey arrows.

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We have no conflicts of interest to declare.

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